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Isolation, sequencing and expression of the superoxide dismutase-encoding gene (*sod*) of *Nocardia asteroides* strain GUH-2

(Molecular cloning; gene library; metal-binding ligand; nucleotide homology; amino-acid homology; double mutant; Western blot; paraquat)

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SUMMARY

Nocardia asteroides (*Na*) superoxide dismutase (SOD) has been implicated as a virulence factor that allows the organism to survive intracellular killing by phagocytic cells. A full-length *Na sod* gene from a pathogenic strain of *Na* (strain GUH-2) was cloned from a recombinant phage library using the *Mycobacterium tuberculosis* (*Mt*) *sod* gene (*Mt sod*) as a probe. The promoter region and structural gene (624 bp) of *Na sod* was sequenced and nucleotide sequence comparisons reveal 77% homology with *Mt sod*. The *Na sod* gene also shares considerable sequence homology with *sod* of other mycobacterial species. In addition, conserved amino acid (aa) sequences important for metal binding indicate that Mn^{2+} is the preferred metal ion ligand for *Na* SOD. An *Na sod* expression plasmid, pYEX1, under transcriptional control of the *Mt hsp70* promoter (pY6013), produced a 25-kDa protein product which showed SOD activity when stained in a native polyacrylamide gel and reacted with rabbit polyclonal antibody specific for *Na* SOD by Western blot. pYEX1, via transformation, was able to complement an *Escherichia coli* double *sodAB* mutant deficient in SOD production in the presence of paraquat (methyl viologen) which stimulates the production of superoxide radicals.

INTRODUCTION

Nocardia asteroides (*Na*) can cause infections in humans that involve multiple organ systems (Beaman and Beaman, 1994). The severity of these infections are more pronounced in immunocompromised individuals, such as those with AIDS. The pathogenic *Na* actively provoke host defenses, but depending on the strain, are highly resistant to killing by phagocytic cells (Beaman and Beaman, 1990; 1994). The inability of macrophages and PMNs to readily destroy *Na* can be attributed to the antioxidant defense system of the organism (Beaman et al., 1985; Beaman and Beaman, 1990; 1994). Superoxide dismutase (SOD) is an essential element of this defense system (Carlioz and Touati, 1986; Fridovich, 1986; Hassett and Cohen, 1989).

SOD is a metalloenzyme that scavenges superoxide radicals that can lead to oxygen toxicity for the organism

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); AIDS, acquired immune deficiency syndrome; BHI, brain heart infusion (broth); bp, base pair(s); *E., Escherichia*; GAP, sequence alignment program; GCG, Genetics Computer Group (Madison, WI, USA); GTG, translation initiation site; GUH-2, *N. asteroides* human pathogenic strain; *hsp70*, heat-shock 70 gene; kb, kilobase(s) or 1000 bp; *Mt, Mycobacterium tuberculosis*; *Na, Nocardia asteroides*; nt, nucleotide(s); OD, optical density; ORF, open reading frame; p, plasmid; PA, polyacrylamide; PCR, polymerase chain reaction; PMN, polymorphonuclear neutrophil; RBS, ribosome-binding site(s); re-, recombinant; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase(s); *sod*, gene encoding SOD; TAA, translation termination site; TN, 25 mM Tris·HCl pH 7.4/0.5 M NaCl; u, unit(s); [], denotes plasmid-carrier state.

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(Beaman et al., 1983; Fridovich, 1986). The action of SOD results in the conversion of superoxide radicals produced by the oxidative metabolic burst of phagocytes to H_2O_2 and O_2 (Fridovich, 1986). It has been shown that the virulent *Na* GUH-2 strain secretes an unique SOD that is a tetrameric protein which binds equal molar amounts of Fe^{2+}, Mn^{2+}, and Zn^{2+} (Beaman et al., 1983). By utilizing monoclonal Ab which specifically neutralize *Na* SOD activity, in vivo experiments demonstrated that this enzyme played a critical role in nocardial resistance to killing within the murine host (Beaman and Beaman, 1990). Understanding the regulation and expression of *sod* may offer insights into the molecular mechanisms of pathogenesis. We report the cloning of *Na sod* from strain GUH-2 and its sequence analysis. Expression of this gene and functional assessment of *Na* SOD in *Escherichia coli* was also performed.

EXPERIMENTAL AND DISCUSSION

(a) Cloning and analysis of *Na sod*

Screening of 10^4 clones from a λEMBL3 genomic library from GUH-2 revealed ten λ clones. λAC19.5 was selected for further subcloning. The 5.5-kb *Bam*HI subfragment, designated pAC155, was shown to hybridize exclusively to a ^{32}P-labeled plasmid encoding the full-length *Mt sod Mt* strain H37Rv that was kindly provided by Dr. Douglas Young of the Medical Research Council, Tuberculosis and Related Infections Unit, RPMS (London, UK). The *Mt sod* clone consisted of a 1.1-kb *Eco*RI-*Kpn*I fragment cloned into pUC19 (Zhang et al., 1991). By additional Southern blot analysis, *Na sod* was localized on a 1.5-kb *Asp*718 fragment from pAC155. This fragment was cloned into the plasmid vector pSP73 and designated pAC1500. A portion of pAC1500 was then sequenced. The complete nt sequence and derived aa translation of *Na sod* is shown in Fig. 1.

The coding sequence for *Na sod* from the proposed start codon (GTG) to the stop codon (TAA), extends 624 nt (nt 112 to 735). The predicted ORF encodes a 207-aa protein (24 865 Da). This corresponds to the previous result (Beaman et al., 1983) that the subunit mass is 25 kDa. The 5′ upstream region from nt 1 to 111 does not suggest a 5′ leader sequence (Dalmadge et al., 1980; Hirst and Welch, 1988) nor a typical *E. coli* consensus promoter region (Harley and Reynolds, 1987); however, a putative RBS-like sequence is located 8 bp from the predicted start codon at nt 112 to 114 (Fig. 1). The high G+C ratio of *Na sod* is consistent with the G+C ratio of the genus *Nocardia* (64–72%) (Beaman and Beaman, 1994).

Previous results by Beaman et al. (1983) indicated that

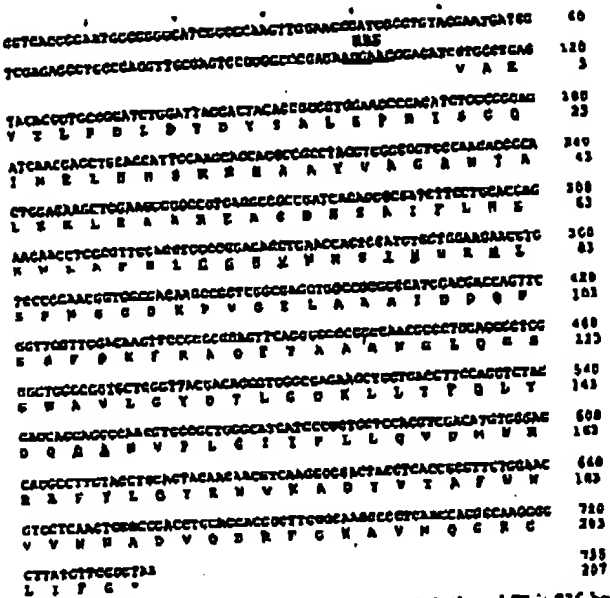

Fig. 1. The nt sequence and translation of GUH-2 *Na sod*. This 735-bp sequence represents a portion of the 1.5-kb *Asp*718 fragment from pAC155. Translation of the nt sequence (from nt 112 to 735) is given below the base sequence using single-letter code designation for aa. A putative RBS is underlined and the proposed start codon GTG is indicated by an arrow. The suspected stop codon is marked with an asterisk (*). Four invariant regions with 3–4 aa, that were identical between SOD sequences from *Na. Mt* (Zhang et al., 1991), *M. leprae* (Thangaraj et al., 1990), human mitochondria MnSOD (Beck et al., 1987), *E. coli* MnSOD (Steinman 1978) and *E. coli* FeSOD (Schinina et al., 1987) when aligned by GeneWorks, are boldfaced. The aa that distinguish between FeSOD and MnSOD enzymes are underlined. Methods: GUH-2 (Georgetown University Hospital, Washington, DC, USA) was isolated from a fatal human infection. Genomic DNA was extracted as described by Brownell and Belcher (1990) with the following exceptions: GUH-2 was inoculated into 1 litre buffered BHI from a 5-day starter culture. After 24 h incubation at 37°C with aeration, glycine (4% final concentration) was added followed by 48 h incubation at 37°C. Then cells were washed and resuspended in 50 mM Tris-HCl pH 8.0/25% sucrose before dry lysozyme was added (3 mg/ml). DNA precipitation, CsCl gradient purification, restriction mapping, subcloning and analysis by Southern blot were performed by standard procedures (Ausubel et al., 1994). The genomic library was generated using *Bam*HI-digested and dephosphorylated λEMBL3 DNA arms (Promega, Madison, WI, USA) according to the manufacturer's instructions. In vitro packaging (Packagene, Promega) of recombinant λ DNA was performed using the methods of the manufacturer. The re-phage library was screened by plaque-lift hybridizations using standard methods (Ausubel et al., 1994). Sequencing was performed by the dideoxy chain-termination (Sanger et al., 1977) method using the Sequenase kit (US Biochemical, Cleveland, OH, USA) and [α-^{35}S]dATP (Amersham, Arlington Heights, IL, USA) according to the manufacturer's protocol. The GenBank accession No. for *Na sod* presented in this report is U02341.

Na SOD bound Mn, Fe and Zn in equimolar amounts as tested by atomic absorption. Parker and Blake (1988) proposed specific aa that could be used to differentiate MnSOD from FeSOD. In particular, aa 76, 77, 79, 83, 84, 87, 154 and 155 (as indicated by underlining in Fig. 1)

are considered the most likely aa for conferring the distinguishing properties. Based on the numbering system in Fig. 1, these aa correspond to 78, 79, 81, 85, 86, 89, 153 and 154, respectively, as proposed by Parker and Blake (1988). *Na* SOD matched the Mn class of SODs for all aa, except 84 and 155, which strongly suggests that the preferred metal ligand is Mn (Fig. 1). Also, the aa specifically designated as ligands to the metal cofactors are conserved for *Na* SOD (data not shown).

(b) Comparative alignment of *sod* genes

Nucleotide sequence comparisons using GAP in GCG (Devereux et al., 1984) between *Na sod* and *Mt. M. leprae*, human (mitochondria, Mn), *E. coli* Mn and Fe *sod* genes revealed similarities of 76.6, 75.3, 57.4, 54.9 and 49.9%, respectively (data not shown). Comparative coding sequence alignments of *Na* SOD with *Mt. M. leprae*, human MnSOD, *E. coli* MnSOD and FeSOD revealed aa homologies of 86, 87, 69, 60 and 55%, respectively, using the GeneWorks program (IntelliGenetics, Mountain View, CA, USA) (data not shown). There are 4 major invariant regions (as indicated by boldface in Fig. 1 with 3–4 aa).

(c) Synthesis and functional activity of *Na* SOD in *E. coli*

The coding sequence of *Na sod* was PCR amplified, cloned between the *Nco*I and *Eco*RI sites of the prokaryotic expression vector pY6013 and designated pYEX1. This directed *Na sod* to be juxtaposed to the *hsp70* promoter allowing a single transcript to be formed. Cell lysates from several transformants were screened for functional SOD activity. Plasmid DNA from one selected clone, pYEX12 (pYEX1 in XL1 host strain), that showed activity (data not shown) was subsequently transformed into the double *sodA sodB* mutant *E. coli* strain QC779 (Δ(*argF-lac*)169, λ⁻, F(*sodB-kan*)1-Δ2, *rpsL*179, *sodA*25::MuPR13, IN(*rrnD-rrnE*)1) (Carlioz and Touati, 1986). Of the ten clones screened for functional activity, all were positive. The SOD activity gel for pYEX125 (QC779 [pYEX1] strain) is shown in Fig. 2. The SOD from both the purified Mn and Fe *E. coli* SOD migrated at an R_f of 0.1 and 0.28 (R_f value relative to the well), respectively (lane 1), while both the *Na* cytoplasmic extract *Na* SOD and the *re-Na* SOD migrated at an R_f of 0.33 (lanes 2, 5 and 6). The *sod*⁺ parent strain of QC779, GC4468 (Carlioz and Touati, 1986), migrated at an R_f of 0.28 (lanes 3 and 8). Of the two positive transformants (pYEX125 and pYEX126; QC779 [pYEX1] host strain) that were screened for complementation of the double mutant in a paraquat growth assay, both showed enhanced growth over that of QC779 (*sodA, sodB*) containing pY6013 only with 0.05 mM paraquat and without (Fig. 3). The parent strain, GC4468, was not affected by paraquat.

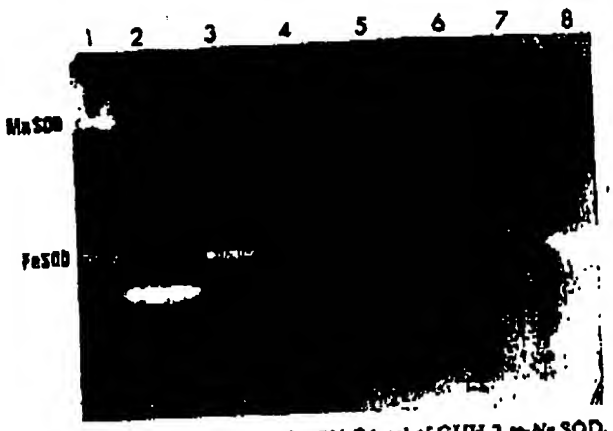

Fig. 2. SOD activity stain on native 8% PA gel of GUH-2 re-*Na* SOD. Approx. 17–110 μg protein was loaded per well. Lanes: 1, MnEC/FeEC (purified *E. coli* Mn and Fe SOD; Sigma, St. Louis, MO, USA); 2, GUH-2 cytoplasmic extract; 3, GC4468/pY6013; 4, QC779/pY6013; 5, pYEX125 (1/2 dilution; pYEX1 in QC779); 6, pYEX125; 7, QC779; 8, GC4468. Methods: The pYEX1 plasmid was constructed from a PCR amplified *Na sod* and the pY6013 plasmid cloning vector kindly provided by Dr. Richard Young (Whitehead Institute, MIT, Cambridge, MA, USA). PCR reactions (50 μl) were performed using Taq DNA polymerase (2.5 units) (Promega) according to standard methods (Innis et al., 1990). Two primers that were designated BB22 (5-GCTGACTGCCATGGTGGCTGAGTACACGCTG) and BB16 (5'-AGCATGGAATTCTTACCCGAAGATAAGGC) were used to amplify *Na sod* from nt 112 to 735. *Nco*I 5' and *Eco*RI 3' sites (underlined), respectively, were included for directional cloning. Reaction conditions were amplified for 30 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C) and extension (1 min at 72°C) with a final extension at 72°C for 5 min. Amplified products were purified from a 1.0% agarose gel by QIAEX (Qiagen, Chatsworth, CA, USA). This expression vector construct (pYEX1) was transformed into *E. coli* XL1 Blue under ampicillin (100 μg/ml) selection. Transformants were screened for *Na* SOD production and function by sonicating (Kontes Micro Ultrasonic Cell Disruptor, Heat Systems, Farmingdale, NY, USA) a washed (TE buffer (0.01 M Tris·HCl, pH 8.0/0.1 mM EDTA)) 1.5 ml overnight culture that was resuspended in 0.25 ml TE. A SOD activity stain (Beauchamp and Fridovich, 1971) was performed as modified by Heinzen et al. (1992) with a few exceptions. Briefly, native 8% PA gels were used, and illuminated for only 10 min using a 60 W bulb. Protein determinations were performed on all sonicates (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA, USA). pYEX1 was electroporated into QC779 (*sodA, sodB*) using standard methods (Ausubel et al., 1994) producing the clones pYEX125 and pYEX126. Control vector were also electroporated (Gene Pulser, Bio-Rad) into QC779 and GC4468.

(d) Immunoblotting

Coomassie blue staining of a 0.1% SDS-12% PAGE performed on pYEX125 sonicate did not result in a discernible 25-kDa band (data not shown). However, when immunoblotting was performed on this sample, as well as *Na* cytoplasmic extract using a purified polyclonal Ab to *Na* SOD, both showed reactivity while the controls,

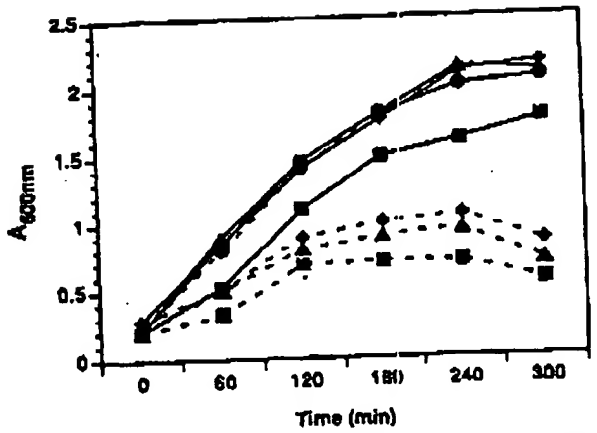

Fig. 3. Complementation analysis of paraquat sensitivity by *Na* SOD production in QC779. Dotted lines denote no paraquat and solid lines denote 0.05 mM paraquat added at $A_{600\,nm} = 0.1$. Symbols: (●) GC4468; (■) QC779/pY6013; (◆) pYEX125; (▲) pYEX126. Methods: Complementation studies were performed as described by Carlioz and Touati (1986) on pYEX125 and pYEX126 to determine their sensitivity to 0.05 mM paraquat. pYEX125 was selected for further analysis by immunoblotting.

QC779[pY6013] and GC4468[pY6013], were negative (Fig. 4).

(e) Conclusions

(1) We have cloned a full-length *sod* from a pathogenic strain of *Na* strain GUH-2 in a re-phage library using *Mt sod* as probe.

(2) The structural *sod* gene (624 bp) of *Na* GUH-2 reveals 77% nt sequence homology and 86% aa sequence homology with the *Mt sod*.

(3) For the 5' upstream region of *Na sod*, there is no evidence of a 5' leader sequence, nor a typical *E. coli* consensus promoter region, however, a putative RBS and a start codon were found by comparison with the known *Mt sod* sequence.

(4) The *Na sod* gene also shares considerable sequence homology with the *sod* genes of other aerobic actinomycetes; however, conserved aa sequences important for metal binding suggest that Mn is the preferred metal ion ligand for *Na* SOD.

(5) The *Na* SOD expression plasmid pYEX1, when transformed into *E. coli*, was able to stimulate growth of the *sod* double mutant deficient in SOD production in the presence of paraquat a stimulant of superoxide radicals production.

(6) Expression of an *Na sod* in *E. coli* produced a 25-kDa protein product that reacted with rabbit polyclonal Ab to purified *Na* SOD by Western blot. This product was a functional SOD as shown by an activity gel.

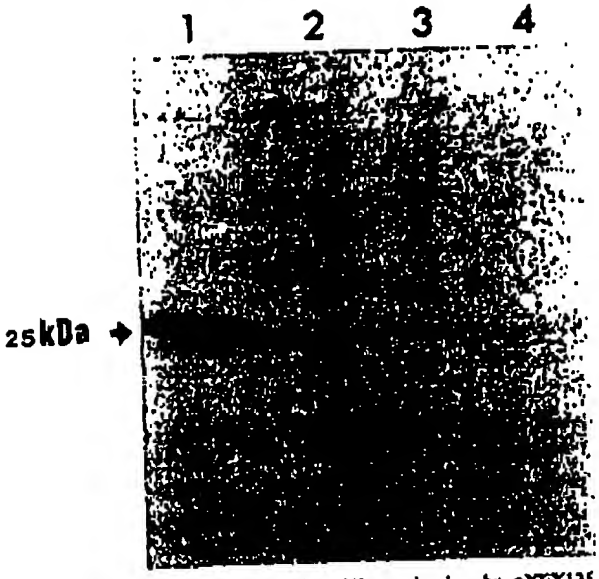

Fig. 4. Western blot analysis of *Na* SOD production by pYEX125. Approx. 20–110 μg protein was loaded per lane. Lanes: 1, *Na* cytoplasmic extract; 2, QC779[pY6013]; 3, pYEX125 (QC779[pYEX1] host strain); 4, GC4468[pY6013]. Methods: 0.1% SDS-12% PAGE was used as described by Laemmli (1970) for Western blot analysis. The gel was transferred using the Trans-blot SD Semi-dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions to ProBlot (Applied Biosystems, Foster City, CA, USA) in 10 mM CAPS pH 11 (3-[cyclohexylamino]-1-propanesulfonic acid) buffer/10% methanol. Immunoblotting was then performed using the ProBlot protocol. The primary Ab was rabbit polyclonal IgG Ab to *Na* purified SOD (Beaman et al., 1985), diluted to 3 ag/ml in TN-milk buffer (25 mM Tris·HCl pH 7.4/0.5 M NaCl/1% skim milk), and the secondary Ab was goat anti-rabbit IgG conjugated to horseradish peroxidase (Fisher Scientific, Pittsburg, PA, USA) diluted 1:1000 in TN-milk buffer.

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